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Why the Rat-1 Fibroblast Should Replace the SCN as the In Vitro Model of Choice

Minireview

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(SCN: your days—and nights—are numbered)

Every once in a while there appears a set of experiments that changes the way a field views itself. This is certainly the case with the Balsalobre et al. (1998) paper published in this issue of *Cell*. The key results were presented by Ueli Schibler at the recent Society for Research on Biological Rhythms Meeting in Amelia Island, Florida (May 6–10), and there was an audible collective gasp in the audience when the principal conclusions took hold. To appreciate fully the importance of this paper, some sense of context may be helpful.

In circadian rhythms, the suprachiasmatic nucleus (SCN) is king (Klein et al., 1991). Discovered approximately 25 years ago, the SCN contains the master oscillator that drives locomotor activity rhythms in mammals. The approximately 10,000 SCN neurons undergo rhythmic oscillations in electrical activity even in dissociated cell culture (Welsh et al., 1995), consistent with the notion that they contain an endogenous cell-autonomous oscillator. These neurons are also central to many studies on the mechanism of entrainment, as they are synchronized by and to the light–dark cycle of the external world. Light is perceived by the retina, and the signal transmitted to the SCN via the retinohypothalamic tract. Glutamate is the major neurotransmitter that communicates light information to the postsynaptic SCN neurons. These are also important for the final part of the rhythm troika, output signaling or how the oscillator connects to its distant targets. It is now known that the SCN contacts the periphery via humoral as well as neural signals (Silver et al., 1996). All of the high-level SCN work has made these neurons the most important players in the field. As a small brain region with an apparently single, dedicated function, this tissue also occupies a deservedly important niche within mainstream neuroscience.

The vertebrate retina is in second place but closing fast. Besharse and colleagues discovered that the *Xenopus* retina contains an autonomous circadian clock which governs the rhythmic synthesis of melatonin in culture (Besharse and Iuvone, 1983). Subsequent studies with mammalian retina confirmed and extended these observations. In vitro retinal cultures are much simpler than comparable procedures with SCN neurons, and there is evidence from the tau mutant hamster that the same fundamental clock is ticking in both key clock tissues (Tosini and Menaker, 1996). The retina's search for a place in the sun is well described by citing the title of a workshop at the recent SRBR meeting referred to above: "Why the Cultured Retina Should Replace the SCN as the In Vitro Model of Choice."

Rhythm work in nonmammalian systems has flowered of late but often within less elegant cell types than retinal and SCN neurons. Photosynthetic bacteria and *Neurospora* house bona fide circadian rhythms (Dunlap, 1996), but the absence of a tight biochemical connection with mammals (the same protein doing the same job) makes it understandable why these lower animal rhythms are not prominently featured within the Society for Neuroscience. *Drosophila* circadian rhythms are less easy to dismiss, as they occur within neuronal as well as nonneuronal cell types (Liu et al., 1988; Siwicki et al., 1988). Moreover, tight molecular connections between fruit fly and mammalian rhythms now exist, thanks in large part to the discovery of mammalian *period* (*mper*) genes (Sun et al., 1997; Tei et al., 1997). Remarkably, at least two *mper* transcripts are expressed and undergo circadian oscillations in level within the SCN. This is similar to the previously described oscillations of *per* transcripts in adult *Drosophila* (Hardin et al., 1990), although there are clear phase differences between the fly RNA rhythms and at least some of the mammalian *per* RNA rhythms. This fly–mouse connection has been further strengthened with the finding of a new *Drosophila* rhythm mutant that encodes *dClock*, the apparent fly ortholog of mouse *Clock* (Allada et al., 1998). This transcription factor probably makes a major and direct contribution to the rhythmic transcription of *per* and its partner gene *timeless* (*tim*).

But the fly story has major elements that appear only marginally relevant to mammalian rhythms. Important among these are the many fly tissues that undergo molecular oscillations (cycling of RNA and protein levels) with little apparent connection to the central oscillator that drives locomotor activity rhythms; indeed, most peripheral cells continue to undergo circadian cycling in the absence of the putative central pacemaker "lateral neurons" (Zerr et al., 1990; Ewer et al., 1992). This led to the suggestion that there are multiple rhythm centers all over the fly. Recent work indicates that these may all be light-responsive and cell-autonomous and therefore contain the same photoreceptor and intracellular signal transduction mechanism that communicates light information to the central clock molecules in the lateral neurons (Plautz et al., 1997). In mammals, autonomous pacemaker tissues are restricted to only a few neuronal tissues of the circadian-neuroendocrine axis, including the retina and the SCN. Importantly, only the eye was known to receive direct photic information in mammals, and the pineal as well as the SCN is downstream of the retinal-hypothalamic tract. Despite the contrast with the widespread circadian photoreception in flies, the highly restricted light input route in mammals is supported by the completely arrhythmic phenotype of totally blind (enucleated) humans (Czeisler et al., 1995). Only the very recent "back of the knee illumination" experiments in people recall the fly situation and are inconsistent with the current mammalian paradigm (Campbell and Murphy, 1998); the functional relevance of this extraocular light input pathway is enigmatic in light of the arrhythmic phenotype of blind humans.

So onto the narrow mammalian SCN-neuronal stage leap Schibler and colleagues along with their rat-1 fibroblasts. They had previously identified a transcription factor family in mammals including two related bZIP proteins called DBP and TEF. DBP (rat albumin D-element-binding protein) was characterized first and discovered to undergo a remarkable circadian oscillation in protein levels, approximately 100-fold in magnitude; the same is true for TEF (Wuarin and Schibler, 1990; Fonjallaz et al., 1996). The protein fluctuations are due in large part to mRNA fluctuations, which are due to circadian regulation of the genes' transcription. These oscillations are not restricted to liver, as comparable oscillations were observed in other mouse peripheral tissues such as lung and kidney, suggesting that one or more systemic regulators under circadian control (e.g., cortisol) drives these oscillations in peripheral tissues. Yet the most recent DBP paper from this laboratory contained a hint that these oscillations may not be totally unrelated to the mammalian central oscillator: robust oscillations also occur in the SCN, and there is a 4 hr phase difference between cycling in the SCN and cycling in the periphery (Lopez-Molina et al., 1997). Moreover, the DBP-knockout mouse showed a small but significant alteration in circadian period compared to the control genotype. The wide tissue distribution of DBP and TEF expression recalls that of mouse Clock, namely, widespread expression in peripheral tissues as well as the brain, with a prominent focus in the SCN. More importantly for this study, it also recalls the wide expression pattern of mper1 and mper2 that accompanies their more well-studied expression in the SCN (Albrecht et al., 1997; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997).

The nearly ubiquitous expression of these clock genes suggested to Schibler and colleagues that one might profit from studies in tissue culture. Indeed, immortalized rat-1 fibroblasts, in cell culture for more than 25 years, also express TEF mRNA (as well as many other circadian-relevant mRNAs—see below) but apparently without any time-of-day regulation. Discovering that these mRNA levels decreased markedly after feeding with serum-rich medium, they astutely followed out the postfeeding time course and realized that mRNA levels not only decreased but returned to high levels. Manipulation of the serum treatment, much longer time courses and the analysis of additional cycling mRNAs indicated that the serum shock resulted in at least three full circadian cycles with an $\sim$22.5 hr period. Periodicity was defined by the waves of mRNA level changes, and every available known in vivo cycling mRNA undergoes an oscillation in the fibroblasts comparable to its in vivo circadian cycle. The phenomenon is limited to circadian RNAs, i.e., other control RNAs show no temporal changes after the serum shock. The cycling mRNAs include mPer1 and mPer2, the rat equivalents, which are expressed in the fibroblasts and cycle with a relative phase relationship identical to what was previously described for the SCN. The tissue culture cycling is not limited to the rat-1 fibroblast line, as a second cell line shows a similar albeit less potent phenomenon, i.e., fewer robust cycles after the serum shock. A number of criteria indicated that the cell cycle is irrelevant; for example, the circadian waves of gene expression were unaffected by Ara-C treatment, which blocks the cell cycle.

In addition to this startling discovery—the presence of a canonical circadian oscillator in established tissue culture lines—there is even more gold in the details. This includes the demonstration that all of these RNAs, including mPer1 and mPer2, undergo an apparently identical cycle in peripheral tissues like liver in vivo. Remarkably, these transcripts had not been previously assayed for circadian changes in levels in peripheral tissues.

Another nugget is the relationship between the serum shock and the more traditional entraining stimulus used in whole animal studies, light. A phase-shifting light stimulus is known to stimulate the transcription of a set of genes within the SCN. Most of the specific SCN light-induced genes are transcription factors, so-called immediate-early genes (IEGs). mPer1 and mPer2 have recently joined this list, as they are also light-inducible (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). Although a definitive experiment is lacking, light-induced gene expression is likely relevant to the SCN timekeeping mechanism. IEGs were originally defined as serum-inducible genes, so it is extremely gratifying that there is now an empirical connection between light-induction in the SCN and serum-induction in culture. In hindsight it is perhaps not surprising that the same genes that are rapidly induced by light in the SCN are also rapidly induced after the serum shock in tissue culture. The tissue culture paradigm also allows application of the acid test for IEG induction, namely, cycloheximide-insensitivity. Indeed, serum induction of mPer1 and mPer2 RNA levels passes this test, indicating that only posttranslational regulation lies between serum and the presumed transcriptional induction of the two genes. The guess is that an SRE (serum response element) may be involved in light induction. One also assumes that IEG activity, including mPer1 and mPer2, then contributes to a subsequent induction or repression of a second wave of clock genes. The ability to transfect the fibroblasts with overexpression or dominant-negative constructs should help establish a more precise role for these two clock proteins within the cycle.

A final fascinating detail includes the fact that all the cycling liver RNAs maintain a 4 hr phase delay relative to the timing program in the SCN. This is identical to the phase delay between SCN and liver originally reported for DBP mRNA and suggests that the SCN and the periphery use different entraining signals: light for the SCN and some circulating molecule(s) for the periphery. Presumably, the circulating molecule undergoes circadian changes in level or activity. By comparing and fractionating serum collected from animals at different times of day, the tissue culture assay should be able to identify the relevant molecule(s). The data also suggest that the SCN is indeed the master clock and controls the activity of the circulating entraining agent. This predicts that an SCN-lesioned animal will lose its peripheral clock cycling. But this experiment is tricky, as RNA levels in each lesioned animal can only be assayed once—at least with traditional methods. This makes it impossible to distinguish between noncycling on the one hand and asynchrony on the other. In the latter case, clocks are

running in peripheral tissues but each animal has a random phase and the population average is noncycling. A more sophisticated assay will be required to distinguish between the two possibilities.

The distinction between asynchrony and noncycling is also relevant to the tissue culture clock prior to the serum shock: has the clock stopped ticking or are the individual cells merely asynchronous? Otherwise put, does the serum shock synchronize the cells or does it induce the cycles to begin de novo? A distinction requires that individual cells be followed in real time pre- and postserum shock, and Schibler stated during his oral presentation that experiments with a DBP-GFP transgene were underway. Also discussed was the possibility that small groups of adjacent cells might be synchronous, which means that autocrine or short-range effectors might contribute to local entrainment and cell (clock) coupling.

If the SCN is responsible for delivering a circadian signal to the peripheral clock, the primacy of this master oscillator is maintained. Moreover, the distinction between the SCN and the driven oscillators in the periphery echos an important, defining feature of true circadian clocks: their ability to free-run in the absence of environmental clues. Although the SCN is also "driven"—by the light–dark cycle—the animals continue to manifest circadian cycles for months in constant darkness. So the current wisdom views light as an entraining agent rather than a driver, whereas a circulating hormone signal might be viewed as driving the peripheral cycles: if the hormone stops oscillating, these cycles should grind to a halt. But the RNA cycling in culture does not stop immediately after the serum shock; on the contrary, it persists for at least three cycles with only marginal damping. Under more favorable culture conditions, who knows how long it might persist? And even if some damping turns out to be an intrinsic feature only of the tissue culture clock, the similarities between the two systems will almost certainly dwarf the differences. This is obvious from the identical phase relationships between the different RNAs, in tissue culture as compared to the SCN. In other words, these driven oscillators are not only free-running (sort-of) but also very similar to the SCN oscillators that are the object of so much focused attention. This recalls the much more democratic view espoused by the current *Drosophila* paradigm: comparable cell-autonomous clocks all over the fly. The prediction from Balsalobre et al. is that the vast majority of the molecular machinery that constitutes the mammalian circadian oscillator—the components and how they tick—will function similarly in the master and slave clocks. So perhaps clocks in neurons and fibroblasts, and in mice and *Drosophila*, are rather similar after all. But you already knew that, now didn't you?

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